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Rχ-01, a New Family of Oxazolidinones That Overcome Ribosome-Based Linezolid Resistance▽

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New and improved antibiotics are urgently needed to combat the ever-increasing number of multidrug-resistant bacteria. In this study, we characterized several members of a new oxazolidinone family, Rχ-01. This antibiotic family is distinguished by having in vitro and in vivo activity against hospital-acquired, as well as community-acquired, pathogens. We compared the 50S ribosome binding affinity of this family to that of the only marketed oxazolidinone antibiotic, linezolid, using chloramphenicol and puromycin competition binding assays. The competition assays demonstrated that several members of the Rχ-01 family displace, more effectively than linezolid, compounds known to bind to the ribosomal A site. We also monitored binding by assessing whether Rχ-01 compounds protect U2585 (*Escherichia coli* numbering), a nucleotide that influences peptide bond formation and peptide release, from chemical modification by carbodiimide. The Rχ-01 oxazolidinones were able to inhibit translation of ribosomes isolated from linezolid-resistant *Staphylococcus aureus* at submicromolar concentrations. This improved binding corresponds to greater antibacterial activity against linezolid-resistant enterococci. Consistent with their ribosomal A-site targeting and greater potency, the Rχ-01 compounds promote nonsense suppression and frameshifting to a greater extent than linezolid. Importantly, the gain in potency does not impact prokaryotic specificity as, like linezolid, the members of the Rχ-01 family show translation 50% inhibitory concentrations that are at least 100-fold higher for eukaryotic than for prokaryotic ribosomes. This new family of oxazolidinones distinguishes itself from linezolid by having greater intrinsic activity against linezolid-resistant isolates and may therefore offer clinicians an alternative to overcome linezolid resistance. A member of the Rχ-01 family of compounds is currently undergoing clinical trials.

The marked increase in infections caused by multidrug-resistant bacteria underscores the urgent need for new agents to combat infections. This need has made the search for new antibacterials a critical, but very challenging, endeavor. Despite this acute need and extensive research, antibiotics from only two new chemical classes of compounds have been approved in the last 30 years: the lipopeptide daptomycin (25) and the oxazolidinone linezolid (4). Oxazolidinones are a class of synthetic antibiotics that originated from a series of oxazolidinones reported to be useful for treating a variety of plant diseases (16). Their antibacterial activities were discovered during the course of a screening program for antibacterials (44). Early on, oxazolidinones were found to exert their actions through the inhibition of protein synthesis (14).

Linezolid (Zyvox), which was approved by the FDA for human use in 2000, has in vitro and in vivo activity against multidrug-resistant gram-positive organisms, such as methicillin-resistant *Staphylococcus aureus*. The first reports of bacterial strains resistant to linezolid started to appear shortly after linezolid's introduction into the clinic (2, 49). Although the number of strains resistant to linezolid is still low (1, 23), there are several recent reports about linezolid resistance involving different clinical settings (10, 38, 41). In almost all cases, resistance to linezolid in a variety of clinical isolates affects the large ribosomal subunit (50S) via a nucleotide mutation resulting in G2576U (*Escherichia coli* numbering) for one or more alleles of 23S rRNA (1, 24).

The link between linezolid resistance and the bacterial 50S ribosomal subunit is supported by a wealth of biochemical studies, which show that the oxazolidinones bind to a site in the ribosome that overlaps the binding sites of lincosamides and chloramphenicol (28, 48). The binding overlap of linezolid with lincosamide antibiotics is also supported by the X-ray structure of linezolid bound to the 50S ribosomal subunit of *Haloarcula marismortui* (20, 45), Protein Data Bank ID code 3CPW. Furthermore, in ribosome function assays, linezolid shows cross-resistance to chloramphenicol (5). Like chloramphenicol, linezolid suppresses nonsense mutations and promotes frameshifting (48). In addition, oxazolidinone-resistant mutants isolated in the laboratory are linked to mutations around the peptidyl transferase region (24, 47, 52).

Using a combination of structural information and computational analysis, we developed a new oxazolidinone family, Rχ-01. This family is effective against drug-resistant bacteria found in community and hospital settings. Members of the Rχ-01 family were designed to have higher affinity for the ribosome than linezolid, thereby overcoming resistant strains and conquering major causative agents in the community, such as streptococci, *Moraxella*, or *Haemophilus*. We used detailed knowledge at the atomic level of the juxtaposition of the ribofunctional loci of linezolid and sparsomycin (15, 20). We identifed an optimal bridging element between these molecules that gives priority to interactions and shape complementarity with the ribosome. Based on the atomic structures of Rχ-01–

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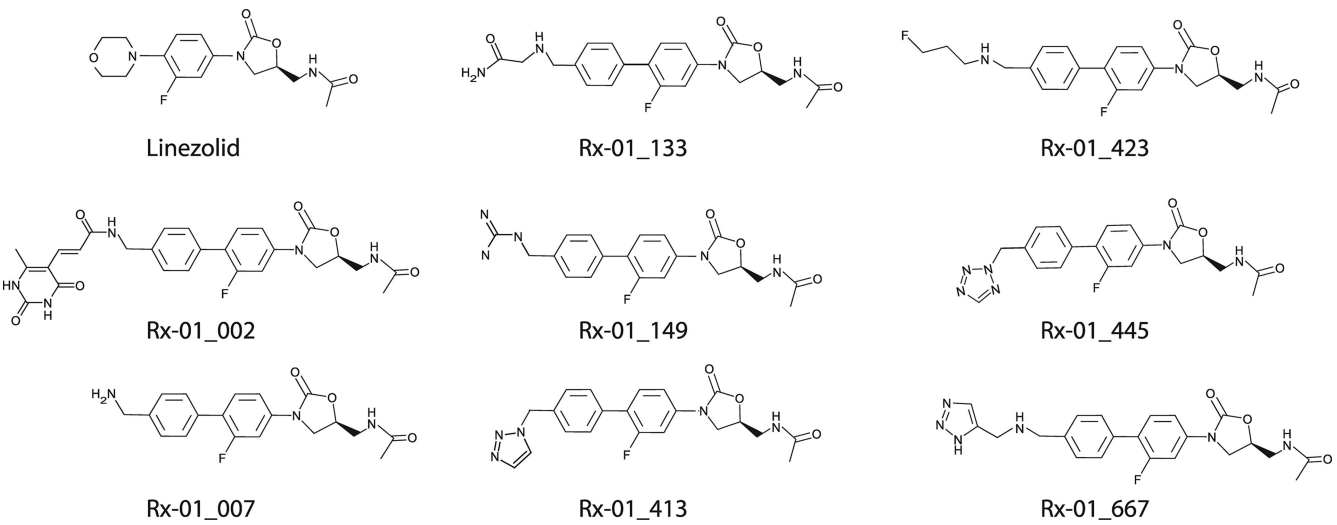

FIG. 1. Chemical structures of Rχ-01 novel oxazolidinones and linezolid.

ribosome complexes, energy analysis, and biological data generated for the initial set of molecules, three key computational models were developed and validated (50). The models were used to improve *H. influenzae* activity, to predict Caco-2 cell permeability (a surrogate for oral absorption) (11), and to predict rat oral bioavailability. The use of this integrated structure-based drug design approach allowed us to identify a new family of oxazolidinones that balanced all features, including potency and desired properties, for this series. These new oxazolidinones form the basis of Rib-X's Rχ-01 program and have led to one compound of the Rχ-01 family entering phase 2 clinical trials.

In our study, several members of the Rχ-01 family of novel oxazolidinones were compared to linezolid. This comparison was based on the Rχ-01 ability to displace chloramphenicol or puromycin from the 50S ribosomes and on the ability of the oxazolidinones to inhibit the translation of 70S ribosomes isolated from linezolid-susceptible and -resistant (G2576U mutation) *S. aureus*. Our study showed that chemical modification of U2585 (*E. coli* numbering), a 23S rRNA nucleotide that influences peptide bond formation and peptide release, was hindered by the presence of Rχ-01 compounds but not by the presence of linezolid. In addition, we also discovered that the Rχ-01 family binds the 50S ribosomal subunit more strongly than linezolid. As a consequence, Rχ-01 compounds, compared to linezolid, show enhanced antibacterial activity and a stronger ability to promote translational inaccuracy. Furthermore, in translation assays, Rχ-01 compounds are able to overcome the ribosomal mutation found in most linezolid-resistant clinical isolates, suggesting that Rχ-01 compounds may be ideal candidates to combat linezolid resistance in the clinic.

MATERIALS AND METHODS

Antibiotics. Compounds Rx-01_002, Rx-01_007, Rx-01_133, Rx-01_149, Rx-01_413, Rx-01_423, Rx-01_445, and Rx-01_667 (9, 34) and linezolid (7) (Fig. 1) were synthesized at Rib-X Pharmaceuticals, Inc., New Haven, CT. Chloramphenicol, gentamicin, and tylosin were obtained from Sigma (St. Louis, MO). [α-^{33}P]dTTP (3,000 Ci/mmol) was obtained from Perkin-Elmer, [^{3}H]chloramphenicol (20 Ci/mmol) was obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO), and [^{3}H]puromycin (9.1 Ci/mmol) was obtained from Moravek (Brea, CA). Etamycin and griseoviridin, used as controls to validate the assay (data not shown), were a gift from G. S. Katrukha, Institute of New Antibiotics, Moscow, Russia.

Bacterial strains. *S. aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were obtained from the American Type Culture Collection. *S. aureus* RN1786, a nuclease-deficient strain and the source of wild-type ribosomes for translation, was obtained from S. Khan (University of Pittsburgh School of Medicine). *S. aureus* A7820 (Lin^{r} Erm^{r}) was derived from A7819, a linezolid-resistant (G2576U) strain, isolated in the clinic, to which the *ermC* methylase gene had been introduced (39) and was obtained from Robert Moellering, Jr. (Beth Israel Deaconess Medical Center, Boston, MA). *E. coli* MC245 strains containing plasmids with *lacZ* protein fusions engineered to test translational accuracy (48) were a generous gift from A. Dahlberg (Brown University, Providence, RI).

Preparation of *S. aureus* 70S ribosomes. Ribosomes were prepared according to the method of Rheinberger et al. (37) with small modifications to adapt the protocol for *S. aureus*. Briefly, an overnight culture of *S. aureus* was used to inoculate fresh tryptic soy broth medium. Bacteria were grown at 37°C to an optical density at 600 nm of 2 and cooled to 0°C to produce runoff (empty) ribosomes. Cells were harvested and frozen in liquid nitrogen. Frozen *S. aureus* cells were resuspended in TMKPL buffer [10 mM Tris, pH 8.2, with acetic acid, 14 mM $Mg(OAc)_2$, 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 units/ml lysostaphin] at 4°C. The cell suspension was lysed by three consecutive passes through an EmulsiFlex-C5 microfluidizer (Avestin, Ottawa, Canada) cell, followed by the addition of DNase I to a final concentration of 1 unit/ml of the cell extract. The supernatant was centrifuged at 30,000 × *g* for 20 min at 4°C. The upper three-fourths of the resulting supernatant was mixed with 1.1 M sucrose (0.24 ml per ml of supernatant) and was centrifuged again at 30,000 × *g* for 2 h at 4°C. The cleared supernatant was loaded on a 1.1 M sucrose cushion and was centrifuged at 100,000 × *g* for 16 h. The ribosome pellet was suspended in TMK buffer and dialyzed for 2 h at 4°C against the same buffer, frozen in liquid nitrogen, and then stored at −80°C. Ribosomes (70S) were prepared from *S. aureus* (Lin^{r} Erm^{r}) in a fashion similar to *S. aureus* wild-type ribosomes except that the cells were grown in medium with erythromycin (50 μg/ml) to ensure a high level of A2058 methylation.

Translation inhibition assays. To test inhibition by Rχ-01 compounds of protein synthesis, we developed several prokaryotic in vitro translation-only assays. We developed these assays by modifying a previously described transcription/translation assay fueled with an *E. coli* S30 extract (Promega part number L1020) (35a). The translation-only assay uses purified *S. aureus* 70S ribosomes (20 nM final concentration) in TMK buffer (10 mM Tris-HCl, pH 7.4, 6 mM MgCl, 60 mM KCl, 1 mM dithiothreitol), various amounts of S100 extracts from different bacterial sources, Promega amino acid mix (0.1 mM final concentration), 3 μl of Promega S30 premix, and 200 to 800 nM (final concentration) of an in vitro-transcribed mRNA encoding firefly luciferase. The final volume of each translation reaction mixture was 10 μl. All compounds were tested in duplicate for translation inhibition, and all assays included both positive and negative

TABLE 1. Translation-inhibitory activities of Rχ-01 compounds

Compound	IC_{50} (μM) of source of ribosomes for translation		Selectivity ratio[a]
	S. aureus QC ATCC 29213	*S. aureus* A7820 (Lin^r Erm^r)	
Rx-01_002	0.09	0.03	17
Rx-01_007	≤0.02	≤0.02	>100
Rx-01_133	≤0.02	≤0.02	>50
Rx-01_149	≤0.02	0.08	>100
Rx-01_413	0.13	0.21	>100
Rx-01_423	≤0.02	0.06	>100
Rx-01_445	0.06	0.06	23
Rx-01_667	0.02	0.03	>100
Linezolid	0.9	8.4	>100
Chloramphenicol	7.8	54	25
Florfenicol	0.7	16.4	>100
Sparsomycin	≤0.02	≤0.02	7

[a] Rabbit reticulocyte IC_{50}/*S. aureus* QC IC_{50}.

TABLE 2. Microbiology activities of Rχ-01 compounds

Compound	MIC (μg/ml)				
	S. aureus QC ATCC 29213	*S. aureus* A7820 (Lin^r Erm^r)	*E. faecalis* ATCC 29212	*E. faecalis* P5 (Lin^r)	*E. faecium* A6349 (VanA Lin^r)
Rx-01_002	0.25	8	1	16	1
Rx-01_007	4	8	0.5	8	1
Rx-01_133	4	8	0.5	8	1
Rx-01_149	2	8	2	8	2
Rx-01_413	0.5	1	0.5	1	1
Rx-01_423	1	8	0.5	2	1
Rx-01_445	0.5	1	0.25	1	0.5
Rx-01_667	1	8	0.25	1	0.5
Linezolid	4	64	4	32	16
Chloramphenicol	8	64	8	32	24
Florfenicol	8	64	4	16	16

controls to measure translation, either in the absence of a compound or in the presence of an antibiotic known to predictably and reproducibly inhibit translation. A Victor2V Multilabel Reader (Perkin Elmer) was used to read luminescence. To obtain compounds selective for prokaryotic ribosomes, we also tested the compounds to inhibit translation fueled by a rabbit reticulocyte lysate (nuclease-treated) system (Promega part number L4960), following the protocol in Promega's technical manual 232 (35b) and using mRNA (200 to 800 nM final concentration) encoding firefly luciferase. Fifty percent inhibitory concentrations (IC_{50}s) were calculated using MDL Assay Explorer with a one-site competition model of binding.

Competitive binding studies. 70S ribosomes from *S. aureus* ATCC 29213 were incubated in ribosomal buffer (10 mM HEPES-KOH, pH 7.8, 10 mM MgOAc, 60 mM NH_4Cl, 6 mM mercaptoethanol) in the presence of either [^{3}H]chloramphenicol or [^{3}H]puromycin and increasing concentrations of unlabeled control antibiotics or Rχ-01 compounds to assess the binding competition. Ribosomes were separated from unbound compounds by spin column chromatography using Bio-Gel P-30 from Bio-Rad equilibrated with TMK buffer. The degree of radioactive chloramphenicol or puromycin displacement was quantified via scintillation counting (53). The apparent IC_{50} was defined as the concentration of compound that displaced 50% of the bound chloramphenicol under fixed non-equilibrium conditions and was calculated using Prism V4 (GraphPad Software Inc.). Etamycin, griseoviridin, and tylosin were used to validate the method (data not shown).

RNA footprinting. To determine whether Rχ-01 compounds protect 23S rRNA bases from chemical modification, 70S ribosomes (50 to 200 nM) were incubated for 10 min at 37°C, followed by 10 min at 20°C in 50 μl of the corresponding modification buffers (46) containing 0.1 to 1 mM antibiotics. 1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulfonate (CMCT) and dimethyl sulfate modifications were carried out at 37°C or at room temperature, as described previously (31). Primer extension was performed in the presence of [α-^{33}P]TTP according to the method of Stern et al. (46) with a set of primers specific for either *E. coli* or *S. aureus* 23S rRNA, depending on the source of ribosomes used in the experiment. Dried gels were exposed and bands were quantified with a "Storm" scanner (Molecular Dynamics).

Translational-accuracy assay. To perform translational-accuracy assays, *E. coli* strains containing plasmids to test translational accuracy (48) were incubated with sub-MIC concentrations of test compounds. In these strains, the level of β-galactosidase activity is dependent on the abilities of the test compounds to promote either a +1/−1 frameshift event or a stop codon readthrough. Each experimental point was generated by averaging the results of three separate assays measured in duplicate (33). *E. coli* MC245 containing one of four different *lacZ* plasmids was used to determine the level of translational inaccuracy caused by Rχ-01 compounds. Two of the constructs allowed us to test for either +1 frameshifting or −1 frameshifting, while the other two constructs allowed us to test for stop codon readthrough of either UAG or UGA stop codons. Because the levels of β-galactosidase produced from each of the reporter plasmid constructs varied in the absence of antibiotic, the values for the zero drug controls were set at one to allow direct comparison between constructs. Reporter cells were grown to an optical density at 600 nm of 0.3 in the presence (12 to 14 h) or in the absence (4 h) of subinhibitory drug concentrations (48).

RESULTS

Rχ-01 compounds overcome ribosome-based linezolid resistance. We compared the ability of the Rχ-01 family of oxazolidinones to inhibit bacterial protein synthesis to that of linezolid in a translation-only assay using ribosomes isolated from two different sources: *S. aureus* ATCC 29213 (wild type) or *S. aureus* A7820 (Lin^r Erm^r). *S. aureus* A7820 has a plasmid carrying *ermC* methylase, as well as a G2576U mutation in each allele of 23S rRNA. Most linezolid resistance in staphylococci and enterococci isolated in clinical settings is due to this G2576U mutation (22, 36, 38, 39, 49, 51). Our results indicated that Rχ-01 compounds inhibit translation fueled by *S. aureus* wild-type ribosomes up to ≥45-fold better than linezolid (Table 1). When translation was mediated by Lin^r Erm^r *S. aureus* ribosomes, there was a 10-fold increase in the IC_{50} for linezolid and only at most a ≤4-fold increase for Rχ-01 compounds compared to wild-type values. Furthermore, the abilities of the Rχ-01 compounds to bind tightly enough to ribosomes to overcome ribosome-based linezolid resistance do not come at the expense of selectivity. Translation assays showed that most Rχ-01 compounds are at least 100-fold less active in inhibiting translation in rabbit reticulocytes than in *S. aureus* ribosomes. Thus, Rχ-01 compounds not only bind more tightly to ribosomes than linezolid, they also display a selectivity ratio comparable to that of linezolid (Table 1).

Antimicrobial activities of Rχ-01 compounds. We determined MICs on linezolid-susceptible and -resistant *S. aureus* and enterococcal clinical isolates bearing the G2576U mutation. Our study showed that all members of the Rχ-01 family were equally or more effective than linezolid in inhibiting the growth of *S. aureus* and *E. faecalis* strains susceptible to linezolid (*S. aureus* QC [Table 2]). When potency at the ribosomal target was assessed by in vitro translation assays, most members of the Rχ-01 family of novel oxazolidinones, in contrast to linezolid, chloramphenicol, and florfenicol, overcame resistance linked to the G2576U mutation. Although the IC_{50}s in translation for *S. aureus* Lin^r Erm^r ribosomes (Table 1) did not always mirror the ranking of Rχ-01 compounds according to their antibacterial activities compared to linezolid-resistant strains (Table 2), in every case the Rχ-01 compounds were more potent than linezolid against linezolid-resistant isolates,

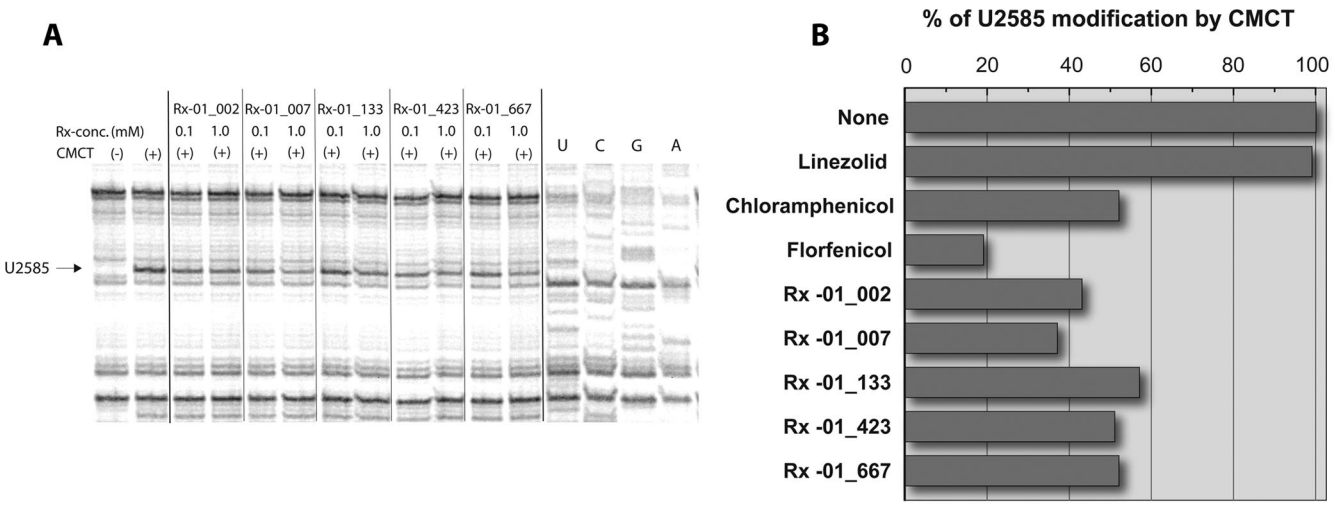

FIG. 2. (A) CMCT modification of 23S rRNA in 70S *S. aureus* ribosomes in the presence of several Rχ-01 compounds at two different concentrations (conc.) (0.1 mM and 1 mM). The results from primer extension shown in the gel correspond to the nucleotide region from 2550 to 2610 in 23S rRNA. (B) Protection of U2585 by Rχ-01 and control compounds. The bar graph was produced by quantifying the relative band intensities of U2585 footprinting in the presence of CMCT from a gel similar to the one shown in panel A.

reflecting their tighter binding to the ribosome. Studies detailing the in vitro potencies versus different community- and hospital-acquired pathogens have already been published (26).

Rχ-01 compounds protect nucleotide U2585 of 23S rRNA. To determine the binding target of Rχ-01 compounds, we also made use of chemical modifications of 23S rRNA. Our study showed that nucleotide U2585 is protected from CMCT modification in the presence of chloramphenicol but not in the presence of linezolid, despite overlapping binding sites (37) (Fig. 2B). Interestingly, nucleotide U2585 is protected from CMCT modification by the binding of Rχ-01 compounds (Fig. 2A and B). The level of U2585 protection attained in the presence of Rχ-01 compounds (40 to 60%) is comparable to that of chloramphenicol (50%) and lower than that of florfenicol (80%). A similar pattern of protection was observed for other compounds that bind to the 50S ribosomal A site (e.g., tiamulin and streptogramin A) (data not shown). These results are in good agreement with the crystallographic structures obtained for various Rχ-01 compounds bound to the 50S ribosomal subunits of *H. marismortui* (21) and confirm that Rχ-01 compounds interact with the ribosome in a manner notably different from that of conventional oxazolidinones.

Determining the structure of linezolid bound to *H. marismortui* 50S required crystals to be soaked in >4 mM linezolid, a concentration that is at least 40 times higher than the concentration used in the chemical protection experiments. The high concentration needed to obtain the structure of linezolid gives additional support to the inability of linezolid, in contrast to Rχ-01 compounds, to protect U2585 at submillimolar concentrations. Figure 3A shows a rendering of the X-ray structure of linezolid bound to the 50S subunit of *H. marismortui* (20) and highlights the position of U2585 (*E. coli* numbering) with respect to the oxazolidinone binding site. Figure 3B shows the positions of linezolid and U2585 with respect to the CCA ends of A- and P-site tRNAs, showing that oxazolidinones, when bound to this pocket, are able to interfere with A-site tRNA positioning.

Rχ-01 compounds have high binding affinity for the ribosome. Structural studies of *Deinococcus radiodurans* 50S have shown chloramphenicol bound at the peptidyl transferase center (40). Nevertheless, subsequent X-ray studies using 50S from *H. marismortui* found a second chloramphenicol binding site at the entrance of the peptide exit tunnel (18). Therefore, we investigated the abilities of Rχ-01 compounds to displace puromycin, in addition to chloramphenicol. Puromycin is the prototypical A-site antibiotic and has been shown by X-ray crystallography to bind in a fashion similar to those of the A site of *H. marismortui* (18, 32) and *D. radiodurans* ribosomes (3).

Because Rχ-01 compounds and chloramphenicol protect U2585 from chemical modification by carbodiimide at about the same level, we assessed if Rχ-01 compounds could displace the A-site inhibitor chloramphenicol or puromycin from its ribosome binding site. The chloramphenicol displacement assay showed that members of the Rχ-01 family of compounds displace chloramphenicol with apparent IC_{50}s 20-fold lower than that for linezolid (Fig. 4A shows Rx-01_007 and Rx-01_423). The abilities of Rχ-01 compounds to displace chloramphenicol and to protect nucleotide U2585 are well correlated, as shown in Fig. 2. Equally, the inability of linezolid to protect U2585 is reflected in its poor ability to displace chloramphenicol from the ribosome. Similar experiments using radiolabeled puromycin instead of chloramphenicol showed that Rx-01_149 was able to displace puromycin with an IC_{50} 100-fold lower than that for linezolid (Fig. 4B). These results suggest that Rχ-01 compounds bind to the ribosomal A site and confirm that they do so with higher affinity than linezolid.

Rχ-01 oxazolidinones cause translational inaccuracy. Linezolid, chloramphenicol, and other inhibitors that bind to the 50S ribosomal subunit promote frameshifting and readthrough of stop codons (28, 48). These effects on translational fidelity may play a significant role in the mechanisms of action of these compounds. Therefore, we investigated whether the Rχ-01 compounds were able to promote translational inaccuracy in a

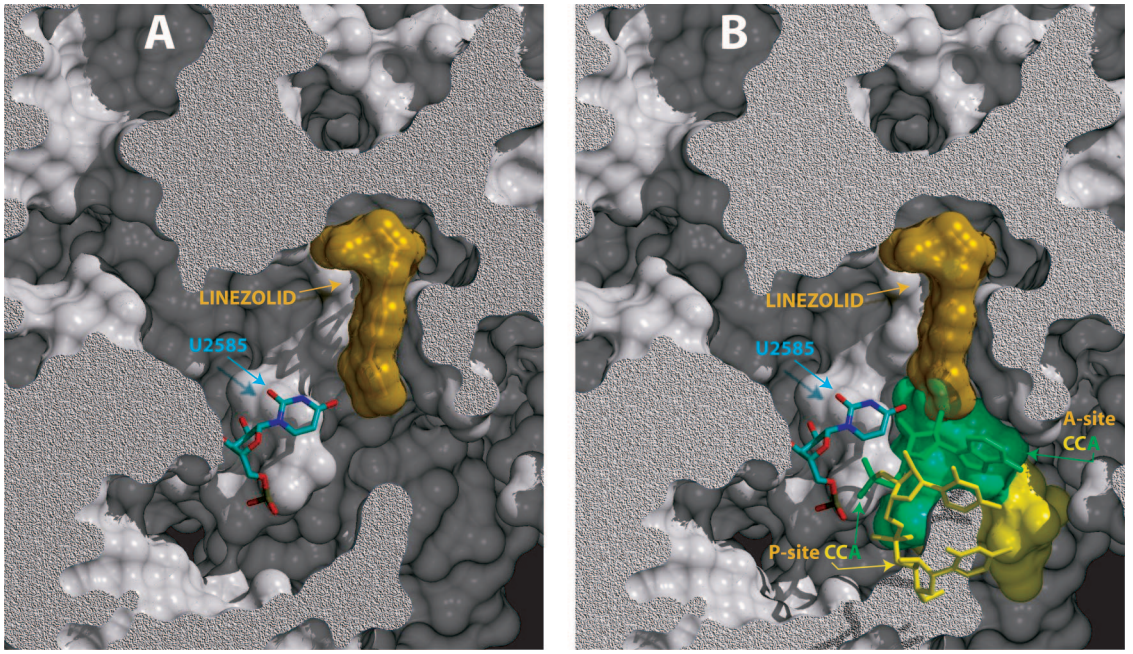

FIG. 3. Longitudinal cut of the 50S subunit of *H. marismortui* showing the structure of linezolid bound to the vicinity of the A site (20). (A) Orientation of U2585 (blue) with respect to linezolid (shown in dark yellow). (B) Position of linezolid with respect to the CCA ends of the A site (surface) and P site (sticks) of tRNA (yellow, C; green, A) (42). The figures were made in VMD (19) and rendered with Raster3D (30).

fashion similar to that of linezolid. We found that Rχ-01 compounds were able to promote stop codon readthrough two- to fivefold more efficiently than linezolid (Fig. 5A). Specifically, Rx-01_423-induced stop codon readthrough is five-fold more efficient than that of linezolid, while Rx-01_667 and Rx-01_413 were three- and twofold more efficient than linezolid. The Rχ-01 compounds Rx-01_413 and Rx-01_423 promoted translational inaccuracy as measured by the promotion of a −1 frameshifting event (Fig. 5B) at extents that were approximately equal to that of linezolid. Rx-01_413 and Rx-01_423 were able to stimulate +1 frameshifting about twofold more efficiently than linezolid, while Rx-01_667 was able to promote both −1 and +1 frameshifting about threefold more than linezolid (Fig. 5B), suggesting that this effect could contribute to their antibacterial activities.

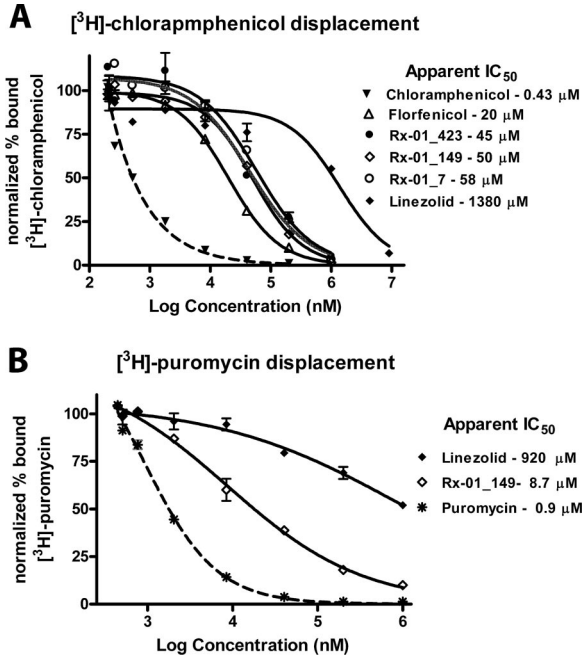

FIG. 4. Displacement of [^{3}H]chloramphenicol (A) or [^{3}H]puromycin (B) from *S. aureus* (wild-type) 70S ribosomal complexes by Rχ-01 compounds and controls. The apparent IC_{50} is defined as the concentration of the compound that displaces 50% of the bound [^{3}H]chloramphenicol or [^{3}H]puromycin under fixed nonequilibrium conditions. The error bars indicate standard deviations.

DISCUSSION

Here, we report that oxazolidinones of the Rχ-01 family bind to the ribosome with higher affinity than the only marketed oxazolidinone antibiotic, linezolid. In translation assays, members of the Rχ-01 family clearly overcame the most common ribosomal mutation (G2576U) associated with linezolid resistance in the clinic. Moreover, the increase in potency seen in the Rχ-01 novel oxazolidinones does not come at the cost of selectivity; the selectivity ratios of Rχ-01 compounds are comparable to those of linezolid (Table 1). Furthermore, the abilities of Rχ-01 compounds to displace chloramphenicol or puromycin to a 20- to 100-fold greater extent than linezolid fit well with the compounds' abilities to bind to the ribosome more strongly than linezolid, as well as with their greater abilities to cause translational inaccuracies.

Initiation complex formation has often been portrayed as the target for oxazolidinone antibiotics (43, 47). However, the ability of oxazolidinones to inhibit initiation complex formation has been observed only at high ratios of oxazolidinones to ribosomes. Moreover, attempts to demonstrate that oxazolidinones inhibit peptide bond formation have not produced clear

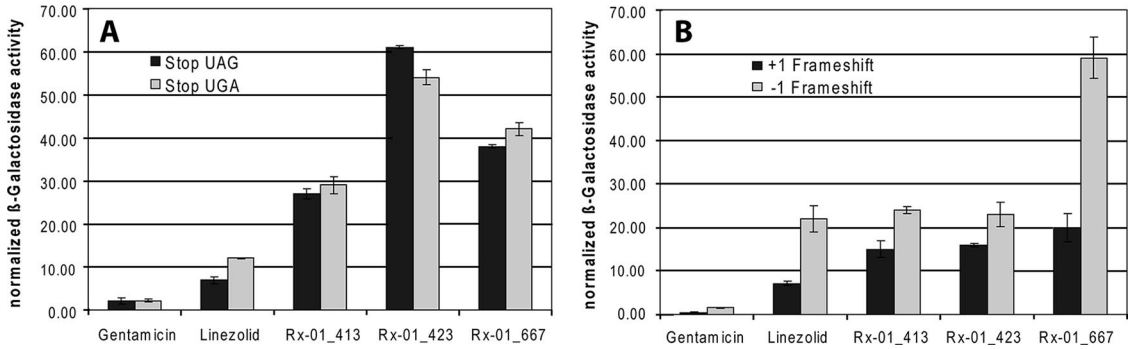

FIG. 5. (A) Production of β-galactosidase by readthrough of stop codons. *E. coli* strains (48) carrying reporter plasmids with either stop codon at the N terminus of the *lacZ* gene were grown in the presence of subinhibitory concentrations of gentamicin (1.5 μg/ml), linezolid (8 μg/ml), Rx-01_413 (0.5 μg/ml), Rx-01_423 (2 μg/ml), and Rx-01_667 (1 μg/ml). (B) Production of β-galactosidase is dependent on frameshifting events. *E. coli* strains carrying reporter plasmids in which the reading frame of the *lacZ* gene contained either a +1 or a −1 frameshift close to the N terminus were grown as described for panel A. Frameshifting and stop codon readthrough values in the absence of antibiotics were set at 1 to allow the relative effects of all antibiotics tested to be compared. The stop codon readthrough and frameshift values obtained for linezolid and for the gentamicin control in this assay were comparable to those obtained by Thompson et al. (48). The error bars indicate standard deviations.

results, except for the inhibition of the first peptide bond as a consequence of the interference of linezolid with the binding of initiator fMet-tRNA(i)(Met) (35). Alternative mechanisms of oxazolidinone action have been proposed when experiments showed that oxazolidinones had a significant in vivo effect on frameshifting and nonsense suppression at concentrations below the MIC (48) and were also able to inhibit 50S ribosomal subunit assembly (8). Clearly, the mechanism of action of oxazolidinones is not fully understood. Nevertheless, like previous publications, this work is consistent with the binding of linezolid to the peptidyl transferase region. The evidence presented here and elsewhere, therefore, provides ample rationale for why all mutations (22, 36, 38, 39, 49, 51) or nucleotide modifications (29) conferring oxazolidinone resistance are located in or close to the peptidyl transferase ring in domain V of 23S rRNA.

Not only do Rχ-01 compounds bind to the ribosome with more affinity than linezolid, they also have at least one additional interaction with the ribosome: they protect nucleotide U2585 from chemical modification (Fig. 2A and B). U2585 is a nucleotide whose CMCT modification has been shown to interfere with binding of peptidyl-tRNA analogs (6), and the rate constants for peptidyl transfer for U2585 mutant ribosomes have been reported to be 10- to 500-fold lower (depending on the mutation) than those of wild-type ribosomes (17). Furthermore, these mutant ribosomes catalyze peptide release at substantially compromised rates (25- to 45-fold) (54). All of these observations underscore the importance of U2585 for efficient peptide bond formation and peptide release (13) and strongly suggest that antibiotics interacting with U2585 will impact the efficiency of protein synthesis.

Rx-01_002, synthesized as proof of concept for the development of the family of Rχ-01 oxazolidinones, was based on the overlap of the binding sites for linezolid and sparsomycin as determined by X-ray crystallography using the *Haloarcula* 50S structures in complex with these two antibiotics (20, 45). We determined that Rx-01_002 inhibits protein translation fueled by wild-type, as well as by linezolid-resistant, *S. aureus* ribosomes and demonstrated that linezolid resistance could be overcome by enhancing binding affinity to the 50S ribosomal subunit (Table 1). The inhibitory effect of Rχ-01 compounds in in vitro protein synthesis translated, in most cases, into whole-cell antibacterial activity against important gram-positive hospital pathogens, including linezolid-resistant enterococci (Table 2) (26).

Based on our findings, we hypothesize that Rχ-01 oxazolidinones affect the rates of peptide bond formation and release because of their stable interaction with U2585. Compared to linezolid, the Rχ-01 oxazolidinones are able not only to inhibit protein synthesis more efficiently, but also to promote translational inaccuracy more effectively. Since decoding is an event controlled by the 30S subunit, the effect of 50S antibiotics on translational fidelity is likely mediated by tRNAs. Therefore, the abilities of oxazolidinones to negatively influence translational fidelity must be a long-range effect mediated by their abilities to perturb or interfere with tRNA binding and/or tRNA positioning at the peptidyl transferase center. Because Rχ-01 oxazolidinones are able to inhibit protein synthesis more strongly than linezolid, our results support the role of translational fidelity as a significant factor in the mechanism of action of oxazolidinones (48).

It has been proposed that oxazolidinones bind to the ribosomal P site (5). Nevertheless, our structural data show that nucleotide U2585 is part of the all-RNA oxazolidinone binding pocket within the ribosomal A site (20). X-ray structures of the 70S ribosome containing tRNA in the ribosomal A site (42) allow us to conclude that binding of Rχ-01 compounds in the A-site pocket could perturb tRNA binding. Our findings are in very good agreement with a recently published model of linezolid bound to the 50S ribosomal subunit generated from in vivo cross-linking data, which indicates that linezolid interacts with the A-site and interferes with the placement of aminoacyl-tRNA (27).

In summary, our structure-based approach successfully yielded the new Rχ-01 family of oxazolidinones, and our biochemical and functional studies helped delineate the mechanism(s) of action of these Rχ-01 compounds. While maintaining its specificity for prokaryotic ribosomes, this family of compounds binds more tightly to ribosomes and is able to inhibit bacterial protein synthesis at concentrations that are

more than 100 times lower than that for linezolid while maintaining its specificity for prokaryotic ribosomes. Thus, our compounds are more potent than linezolid against strains with ribosome-based linezolid-resistant mutations. The new Rχ-01 oxazolidinone family also has a broader spectrum of microbiological activity, including fastidious gram-negative and intracellular pathogens. Studies detailing the in vitro potencies versus different community- and hospital-acquired pathogens, including fastidious gram-negative and intracellular pathogens, have been partially presented and published (12, 26).

Overall, our results indicate that the Rχ-01 family of compounds may be well suited to provide new potent antibiotics effective in the clinic against resistant bacteria. A member of the Rχ-01 family of compounds is currently undergoing clinical trials.

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